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The hypoxia-induced microRNA-130a controls pulmonary smooth muscle cell proliferation by directly targeting CDKN1A.

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Abbreviations:

HPAEC	human pulmonary artery endothelial cells
HPASMC	human pulmonary artery smooth muscle cells
miRNA	microRNA
BMPR2	bone morphogenetic protein receptor type II
CDKN	cyclin dependent kinase
LNA	locked-nucleic acid

Abstract

Excessive proliferation of human pulmonary artery smooth muscle cells (HPASMC) is one of the major factors that trigger vascular remodeling in hypoxia-induced pulmonary hypertension. Several studies have implicated that hypoxia inhibits the tumour suppressor p21 (CDKN1A). However, the precise mechanism is unknown.

The mouse model of hypoxia-induced PH and *in vitro* experiments were used to assess the impact of microRNAs (miRNAs) on the expression of CDKN1A. In these experiments, the miRNA family miR-130 was identified to regulate the expression of CDKN1A. Transfection of HPASMC with miR-130 decreased the expression of CDKN1A and, in turn, significantly increased smooth muscle proliferation. Conversely, inhibition of miR-130 by anti-miRs and seed blockers increased the expression of CDKN1A. Reporter gene analysis proved a direct miR-130 – CDKN1A target interaction. Exposure of HPASMC to hypoxia was found to induce the expression of miR-130 with concomitant decrease of CDKN1A. These findings were confirmed in the mouse model of hypoxia-induced pulmonary hypertension showing that the use of seed blockers against miR-130 restored the expression of CDKN1A.

These data suggest that miRNA family miR-130 plays an important role in the repression of CDKN1A by hypoxia. miR-130 enhances hypoxia-induced smooth muscle proliferation and might be involved in the development of right ventricular hypertrophy and vascular remodeling in pulmonary hypertension.

Keywords
Pulmonary Hypertension, microRNA, seed blockers, hypoxia, proliferation, vascular remodeling

1. Introduction

Pulmonary hypertension (PH), a severe condition leading to right heart failure, is characterized by vasoconstriction, microthrombotic events and remodeling of the small pulmonary arteries [1]. Of these changes, vascular remodeling due to the proliferation of endothelial and vascular smooth muscle cells is arguably the most important alteration and, to date, is not directly targeted by treatment. Several mechanisms have been identified that trigger the development of a pro-proliferative phenotype of these vessel cells, in particular disrupted signaling of the bone morphogenetic protein receptor type II (BMPR2) [2, 3].

Recently, activation of the tumor suppressor p53 in lungs by Nutlin-3a has been shown to prevent and reverse pulmonary arterial remodeling in experimental models for PH [4]. The pro-senescent and anti-proliferative effects of Nutlin-3a were abolished in p21-deficient animals, in which the gene for the cell cycle dependent kinase inhibitor (CDKN)1A (alias p21) was abolished. These genetically modified animals developed significantly more severe pulmonary hypertension than wild-type controls when exposed to chronic hypoxia, which is one of the best-established pathogenic factors triggering pulmonary arterial vasoconstriction and vascular remodeling in an experimental setting [5]. CDKN1A thus appears to be of major importance for mediating the remodeling of pulmonary arteries in response to hypoxia. The mechanisms resulting in altered expression of CDKN1A, however, are unknown. For example, it remains unraveled whether the dysregulation of these signaling events is only a downstream consequence of changes in the expression of BMPR2 or whether other factors might directly target CDKN1A. Such factors might involve the action of microRNAs (miRNAs), i.e. small, non-coding RNAs that bind to the mRNA of a target gene and, by mRNA degradation or inhibition of translation, result in gene silencing. It is suggested that more than 60% of the human genome is regulated by miRNAs [6]. In the context of PH, miRNAs have emerged as important pathogenetic factors [7, 8, 9] that mediate intracellular signaling events [10] and act as post-transcriptional regulators for the expression of BMPR2 [11, 12, 13, 14].

In the current study, following a screening for miRNAs that potentially bind CDKN1A, we focused on members of the miRNA family miR-130 (including miR-130a/b and miR-301a/b) and addressed the effects of these miRNAs on the expression of CDKN1A and, in turn, on the proliferation of human pulmonary artery smooth muscle cells (HPASMC). Finally, we tested the hypothesis whether the use of anti-miRs or locked nucleic acid (LNA) seed blockers directed against miR-130 results in increased expression levels of CDKN1A *in vitro* and *in vivo*.

2. Material and Methods

2.1. Animal experiments

Male mice (C57BL/6) were bred at the Institute for Veterinary Physiology, University of Zurich. The animals were randomly grouped in a normoxic control group (7 mice) and in a hypoxic group (8 mice, 10% O_2). Hypoxic condition was provided in a chamber connected to a gas mixer (Ruskinn Technology Limited, Bridgend, UK). Mice were assessed daily for activity and well-being. Food and water was provided *ad libitum*. Mice employed during initial screening for expression of miRNAs and CDKN1A (Figure 1 and Figure A.3) were exposed to 10% O_2 for 3 weeks, additional kinetic experiments were performed in 3 mice after 2 weeks. At day 21 mice were anaesthetized with 5% isoflurane in oxygen and subsequently euthanized by CO_2 inhalation. Mice used for treatment studies (Figure 6) were exposed to hypoxia for 35 days. LNA seed blockers (2.5mg/kg bodyweight) were injected intraperitoneally twice at day 21 and day 28. At day 35 mice were anaesthetized with 5% isoflurane in oxygen and subsequently euthanized by CO_2 inhalation. Lungs and hearts were collected for further analysis. All animal experiments were approved by Zurich Canton's Veterinary office (approval number 151/2012).

2.2. Cell culture

Human pulmonary artery smooth muscle cells (HPASMC) and human pulmonary artery endothelial cells (HPAEC) were obtained from Gibco (Life Technologies, Zug, Switzerland) and were cultured in supplemented medium 231 and supplemented medium 200 (both from Gibco), respectively. Human embryonic kidney (HEK)293 cells were grown in Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/F12, Gibco) supplemented with 10% fetal calf serum, 10U/ml penicillin, 10µg/ml streptomycin, and 10mM HEPES (all reagents provided by Gibco). Human cells were cultured at 37°C in a humidified atmosphere of 5% CO_2. For experiments in a hypoxic environment, HPASMC and HPAEC were cultured in a humidified atmosphere of 1% O_2.

2.3. Anti-miRs and LNA seed blockers design

Antisense oligonucleotides (anti-miRs) targeting human miR-130a or human miR-301a were synthesized at Microsynth (Microsynth, Balgach, Switzerland). Increased RNA stability was achieved by methylation of its free 2'-hydroxy-group and by introducing six phosphothioate linkages as described by Krutzfeld *et al.* [15]. The sequences are the following: anti-miR-130a (5' – A_SU_SG CCC UUU UAA CAU UGC $A_SC_SU_S$ G_S – 3'), anti-miR-301a (5' – G_SC_SU UUG ACA AUA CUA UUG CA_SC_S U_sG_s – 3') and as a negative control anti-miR scrambled (5' – G_SA_SC CGU UCA CUA UUA CGA GU_SC_S A_SA_S – 3'). For inhibition of the entire miRNA family miR-130, seed blockers comprising the reverse complementary sequence of the miRNA seed match were designed [16]. Efficient binding of these short oligonucleotides (octamers) to their corresponding miRNAs was achieved by using the locked nucleic acid (LNA) design. LNA seed blockers were obtained from Exiqon (Exiqon, Vedbaek, Denmark). The following LNA sequences were used: LNA anti-miR-130 (5' – AUU GCA CU – 3') and as a negative control LNA scrambled (5' – TCA TAC TA – 3') as provided in Figure A.6.

2.4. Quantitative real time-PCR (qPCR) analysis

Total RNA of cultured cells or murine lung tissue samples was purified using the miRNeasy kit (Qiagen AG, Hombrechtikon, Switzerland). Isolated RNA was reverse transcribed by using random hexamers and MultiScribe reverse transcriptase (both from Life Technologies). Quantification of specific gene transcripts was performed by SYBR Green qPCR (Applied Biosystem 7500 system, Life Technologies). Sequences of primers used in this study are shown in the table A.1. Specific amplification was verified by performing melt curve analysis. Obtained expression levels of genes of interest were normalized to the expression of β-actin. Differential gene expression was calculated with the threshold cycle (C_t) method [17].

Quantification of mature microRNAs

Total RNA including miRNA fraction was extracted as described above. Mature miRNAs were detected by specific stem-loop primers, reverse transcribed using MultiScribe reverse transcriptase and quantified by performing SYBR Green qPCR [18]. Primers used in this study for reverse transcription and amplification of miRNAs are provided in the table A.1. Obtained signals were normalized to the expression of snoRNA202 (murine control), RNU48 or RNU49 (both human controls). Specific amplification was confirmed by performing melt curve analysis.

2.5. Western blot

For protein extraction, harvested cells and murine lung tissue samples were lysed with sample loading buffer (62.5mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5mM β-mercaptoethanol, bromphenolblue). Whole-cell lysates were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins were transferred to nitrocellulose membrane (Whatman, GE Healthcare Life Sciences, Little Chalfont, UK). Membranes were incubated with the following primary antibodies: anti – CDKN1A (samples of human origin: rabbit antibody, #2947, Cell Signaling Technology, Danvers, MA, USA; samples of mouse origin: mouse antibody, sc-6246, Santa Cruz Biotechnology, Inc., Dallas,

TX, USA), and anti – β-actin (mouse antibody, #A2228, Sigma-Aldrich Chemie GmbH). Bands were detected with species-specific secondary antibodies coupled to horseradish peroxidase (Jackson ImmunoResearch Europe Ltd, Suffolk, UK). Calculation of the expression of proteins was performed using Adobe Photoshop CS5.1 software (Adobe Systems Incorporated, San Jose, CA, USA) via pixel quantification of the electronic image.

2.6. Plasmid construction

To verify a direct interaction between miRNAs and their predicted targets, the 3' untranslated region (UTR) of the gene of human CDKN1A (1560bp) was amplified from human genomic DNA (Promega AG, Dübendorf, Switzerland) using conventional PCR. The obtained PCR product was double-digested with *Xba*I and *Sal*I (all restriction enzymes from New England Biolabs, Ipswich, MA, USA) and cloned into the luciferase expressing pmiRGLO vector (Promega). In addition to the wildtype (WT) sequences, the predicted miR-130 binding site located in the 3'UTR of CDKN1A and was mutated by site-directed mutagenesis PCR as described in [19]. Primer sequences used for cloning and mutagenesis are shown in table A.1. The correct sequence of the insert was confirmed by sequencing.

2.7. Reporter gene assay

HEK293 cells were seeded in 12-well plates at a density of 50.000 cells per well. The next day, cells were transfected with 200ng of pmiRGLO (either CDKN1A_WT or CDKN1A_ΔmiR130) using Lipofectamine 2000 (Life Technologies). Moreover, molecules for inhibition (anti-miRs or LNA seed blockers) or overexpression (pre-miRs, Life Technologies) of miR-130a or miR-301a (with a final concentration of 25nM) were added. After 24h, the cells were lysed, and *Firefly* luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). The values obtained were normalized to the activity of *Renilla* luciferase.

2.8. Transient transfection of HPASMC

For manipulation of endogenous levels of miR-130, HPASMC were transfected with pre-miR or LNA seed blocker (each 25nM) using Lipofectamine 2000. Silencing of the expression of CDKN1A was achieved by transfecting HPASMC with small interfering RNA (siRNA, 25nM) targeting the coding sequence of CDKN1A (validated siRNA from Qiagen, SI00299810) using Lipofectamine 2000. AllStars negative control siRNA (Qiagen) served as scrambled negative control. Following an incubation period of 24h, 48h or 72h cells were harvested and gene expression analysis was performed.

2.9. Proliferation and apoptosis assay

To assess the proliferation rate, HPASMC were seeded in 96-well plates at a density of 5000 cells per well and were transfected with pre-miRs, anti-miRs or LNA seed blockers (all for the manipulation of the endogenous expression of the miRNA family miR-130, 25nM) using Lipofectamine 2000. After 48h, 5-bromo-2-deoxy-uridine (BrdU) was added and incubated for 24h. Incorporation of BrdU was detected using the colorimetric BrdU assay from Roche (Roche Diagnostics, Mannheim, Germany) and spectrophotometrically measured at a test wavelength of 450nm (reference wavelength: 630nm). The measured absorbance directly correlates with the proliferation and the number of cells. Cell viability was tested using the MTT-based CellTiter assay (Promega) according the manufacture's protocol. Apoptosis was assessed by measuring the activity of caspase 3 and 7 (Caspase-Glo 3/7 assay, Promega).

2.10. Statistics

For statistical analysis, GraphPad Prism Software (GraphPad Software, San Diego, CA, USA) was used. Parametric or non-parametric distribution of data was determined using the Kolmogorov-Smirnov test. The paired or unpaired t-test (two-tailed) as a parametric method for testing was applied. For comparing of more than two sample groups the one-way analysis of variance with Bonferroni *post hoc* test (parametric distribution of data) or Kruskal-Wallis test with Dunns *post hoc* test (non-parametric distribution of data) was used. Values

of $p<0.05$ were considered to be statistically significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). All data are shown as mean ± SD.

3. Results

3.1. The miR-130 family is induced by hypoxia

Computational screening (www.targetscan.org [20]) for miRNAs that target the 3'UTR of CDKN1A retrieved several miRNAs, of which the functionally related miR-130a/b and miR-301a/b were, at least in terms of therapeutic approaches, found most interesting since this family of four different miRNAs might potentially be targeted by one single LNA seed blocker [16]. These four miRNAs were then tested in an experimental model for PH (Fig. 1): upon exposure to hypoxia, mice developed PH as indicated by planimetric increase of the right ventricular volume (Fig. 1a). In lung tissue obtained from these mice, miR-130a (2.61±0.35 vs. 3±0.17, $p=0.02$, lower Ct values indicate higher expression of the miRNA of interest, Fig. 1b), miR-130b (7.93±0.45 vs. 8.37±0.48, p=ns, Fig. 1c), miR-301a (7.91±0.18 vs. 8.2±0.19, $p<0.01$, Fig. 1d) and miR-301b (8.16±0.2 vs. 8.73±0.21, $p<0.001$, Fig. 1e) were significantly upregulated when compared to normoxic control animals. Kinetics of miR-130a expression and concomitant hemodynamic measurement after 2, 3 and 5 weeks are shown in Appendix Figure A.1.

These data were confirmed *in vitro* by using HPASMC, which, under hypoxic conditions (1% O_2 for up to 72h), showed a time-dependent trend toward upregulation of miR-130a and significant upregulation of miR-301a after 72h (1.56±0.19 fold change, $p<0.01$, Fig. A.2a) as compared to normoxic controls. No change of the expression levels was observed in human pulmonary artery endothelial cells (HPAEC, Fig. A.2b). These data suggest that HPASMC are the main source for overexpressed miR-130 family within the lungs.

3.2. Transfection of miR-130a and miR-301a increases proliferation of HPASMC

In a next step, HPASMC were transfected with two representative miRNAs derived from the miR-130 family, i.e. pre-miR molecules for miR-130a, miR-301a and a combination of both and proliferation was assessed using the BrdU incorporation assay. As shown in Figure 2a, overexpression of miR-130a increased proliferation of HPASMC as compared to scrambled transfected cells (in absorbance units at 450nm: from 0.43±0.2 to 0.62±0.2, $p=0.06$). Similarly, transfection of HPASMC with pre-miR-301a resulted in enhanced proliferation (from 0.43±0.2 to 0.75±0.24, $p<0.01$). A "cocktail" transfection using both pre-miRs had no additive effect but resulted in significant increase of proliferation as compared to scrambled transfected cells (from 0.43±0.2 to 0.66±0.19, $p=0.043$). Proliferation data were confirmed by using the MTT-based cell viability assay (Fig. A.3a). No effects were seen on apoptosis (Fig. A.3b).

Conversely, inhibition of miR-130 family reduced proliferation of HPASMC significantly. Anti-miRs specifically directed against miR-130a (from 0.68±0.3 to 0.37±0.24, $p<0.01$), miR-301a (from 0.68±0.3 to 0.25±0.2, $p<0.001$) or concomitant use of both anti-miRs (from 0.68±0.3 to 0.25±0.15, $p<0.01$) decreased proliferation significantly (Fig. 2b). This effect was found to be enhanced by the use of LNA_anti-miR-130 (a seed blocker directed against the seed sequence of miR-130 that blocks all members of the miR-130 family) resulting in decreased proliferation from 0.48±0.24 to 0.17±0.07 ($p<0.01$, Fig. 2c).

3.3. Members of the miR-130 family regulate the expression of CDKN1A

Since CDKN1A is a major regulator of the cell cycle involved in proliferation [21], the expression of CDKN1A was investigated in HPASMC. In HPASMC, the transfection of pre-miRs significantly decreased the expression of CDKN1A, both on mRNA and protein levels (Fig. 3a and 3b). In detail, transfection of pre-miR-130a caused a decrease on mRNA (0.71±0.07 fold change, $p<0.01$) and on protein levels (ratio to β-Actin: from 0.91±0.15 to 0.66±0.16, $p=0.027$) as compared to scrambled controls. Overexpression of pre-miR-301a reduced the levels of CDKN1A by 0.61±0.08 fold change ($p<0.01$) on mRNA and from

0.91±0.15 to 0.65±0.2 (p=0.049) on protein levels. A “cocktail” transfection had no additive effect but resulted in significant decrease of mRNA levels of CDKN1A.
Conversely, the use of LNA seed blockers directed against miR-130 increased the expression of CDKN1A significantly as compared to untreated controls, an effect observed both on mRNA and protein levels: LNA seed blocker directed against miR-130 increased the expression of CDKN1A by 1.46±0.22 fold (p=0.024) on mRNA level (Fig. 3c) and from 0.62±0.19 to 1.07±0.18 (p<0.01) on protein level (Fig. 3d). Successful alteration of miR-130 expression in HPASMC was proven by qPCR analysis and is provided in Figure A.4.
Reporter gene constructs of the entire 3’UTR of CDKN1A (Fig. 4a) were employed to investigate whether the interaction between miR-130a and miR-301a and CDKN1A is direct or indirect. In the wild-type construct, addition of miR-130a (0.84±0.09 fold change, p<0.01) and miR-301a (0.85±0.17 fold change, p=0.045) resulted in significant reduction of relative luciferase activity, whereas insertion of specific point mutations in the miR-130 seed match of CDKN1A abolished the miRNA-induced reduction of luciferase activity (Fig. 4b). *Vice versa*, treatment with LNA seed blockers increased the relative luciferase activity of the wild-type construct by 1.12 fold change (p<0.01) when compared to scrambled controls. No effects on the luciferase activity were observed in the mutated construct (Fig. 4c).

3.4. Silencing of CDKN1A by siRNA mimics the effects of miR-130a and miR-301a
As a proof of principle, siRNA targeting the mRNA of CDKN1A (si_CDKN1A) was performed. As shown in Figure 5a and 5b, expression of CDKN1A was significantly reduced on mRNA (0.42±0.07 fold change, p<0.001) and protein levels (from 1.23±0.13 to 0.64±0.07, p<0.001) when cells were treated with si_CDKN1A. Proliferation of HPASMC was found to be significantly increased when assessed by the BrdU incorporation assay in cells treated with si_CDKN1A compared to scrambled negative control (from 0.47±0.24 to 0.66±0.3, p=0.042, Fig. 5c).

3.5. Hypoxia reduces the expression of CDKN1A *in vitro* and *in vivo*
The effects of hypoxia on the expression of CDKN1A were tested *in vitro* by using HPASMC and *in vivo* in lung tissue obtained from mice that were kept for 3 weeks under hypoxic conditions (10% O_2) and developed PH. Levels of CDKN1A mRNA in HPASMC were found to be significantly reduced at different time points from 8h (by 0.69±0.05 fold change, p<0.01) to 72h (by 0.77±0.08 fold change, p<0.01, Fig. A.5a). These findings were confirmed *in vivo* showing decreased mRNA levels of CDKN1A (by 0.81±0.2 fold change, p=0.03, Fig. A.5b) in hypoxic mice when compared to normoxic control animals.

3.6. LNA seed blockers against miR-130 restore the expression of CDKN1A and ameliorate right ventricular hypertrophy
The therapeutical effects of inhibiting miR-130 were investigated by the use of LNA seed blockers, which were injected intraperitoneally twice in hypoxic animals (Fig. 6a). Planimetric analysis of the ratio between the right and left ventricular volume (Fig. 6b) showed that the differences between normoxic controls (0.25±0.04) and hypoxic animals (LNA scrambled, 0.40±0.07) were no longer significant after treatment with LNA seed blockers (0.35±0.04) indicating an improvement of right ventricular hypertrophy. The expression of CDKN1A was found significantly increased in lung tissue after injections of LNA 130 (Fig. 6c).

4. Discussion

Herein we showed that major members of the miRNA family miR-130 are upregulated in the murine model of hypoxia-induced PH, that, upon induction by hypoxia, regulate proliferation in HPASMC by directly targeting the tumor suppressor CDKN1A (p21). In addition, we demonstrated that a novel generation of miRNA-inhibiting compounds, i.e. LNA seed blockers, are a feasible approach to efficiently counteract the activity of the miR-130 family. These data imply an important role of miR-130 in the pathogenesis of pulmonary vascular remodeling, which, to date, remains an untreated condition.

Proliferation of HPASMC is an essential feature that triggers the development of vascular remodeling, which, itself, is the most important factor in the pathogenesis of PH. The factors that induce a pro-proliferative phenotype in these cells are not completely understood.

The most important factor identified in remodeling of pulmonary arteries is BMPR2, which, when mutated or dysregulated, results in a pro-proliferative state of endothelial and smooth muscle cells. miRNAs of the cluster miR-17/92 have been implicated to silence BMPR2 [11], [22]. Since miRNAs act in a pleiotropic manner, i.e. one specific miRNA might have many gene targets whereas one gene might be targeted by many different miRNAs, we tested whether miR-130 binds the 3'UTR of BMPR2. Although predicted by software, miR-130a and miR-301a appeared to have no direct effects on the expression of BMPR2 in HPASMC (data not shown).

Hypoxic conditions due to pulmonary disorders result in the elevation of the pulmonary pressure and are used to mimic human disease in experimental models. Hypoxia induces proliferation of vascular smooth muscle cells, probably mediated by downregulation of CDKN1A [23]. In another study, this pro-proliferative and anti-senescent effect was enhanced in animals lacking CDKN1A [4]. Since miRNAs are an emerging group of factors that regulate gene expression, we performed an *in silico* approach to identify miRNAs that potentially target CDKN1A. Several miRNAs were retrieved and tested, among which four members of the miR-130 family were found to be induced by hypoxia in lung tissue and HPASMC. Of interest, a very recent study has elegantly shown that the miR-130 family heads a network of miRNAs that regulates proliferation in PH [24]. Our findings confirm these data and previous reports on miRNA analysis in experimental hypoxia-induced PH that found miR-130a/miR-301a significantly upregulated [25]. Moreover, miR-130 was already implicated in the proliferation of vascular smooth muscle cells in systemic hypertension [26] and was associated with the development of airways and lung vessels [27].

Bertero *et al.* suggested a role of miR-130 for controlling the expression of CDKN1A [24]. However, the question remained unanswered whether miR-130 itself or one of the subordinated miRNA pathways affects this master regulator of proliferation. Moreover, it was unclear whether the effects observed were the consequence of a direct miRNA-mediated interaction or whether other factors are involved. Our data here, employing reporter gene studies with constructs of the 3'UTR of CDKN1A (wildtype and mutated miR-130 seed match) identified a direct interaction of miR-130 family with the transcript of CDKN1A. These findings highlight the importance of miR-130 in pulmonary vascular remodeling and have further impacts for our understanding of the pathogenesis of the disease and the development of causative therapies. The most commonly described pathway that regulates the expression of CDKN1A involves the tumor suppressor p53, probably activated by the hypoxia-induced factor 1 alpha (Hif-1α), that acts upstream of CDKN1A [28, 29]. In these studies, however, hypoxia resulted in upregulation of CDKN1A and, in turn, cell cycle arrest. In contrast to these findings, our data emphasize an alternative signaling mechanism involving the hypoxia-induced miR-130a and miR-301a that bind CDKN1A and suppress its expression independent of p53-responsive elements. The contradictory results in our and previous studies are not fully explained at this moment, but might, similar to the paradoxical vasoconstriction induced by hypoxia, represent a unique feature of pulmonary vessels. Since the p53 activator Nutlin-3a required the presence of CDKN1A to improve hemodynamics and prevention of remodeling in hypoxia-induced PH [4], we suggest that CDKN1A, rather than p53, is the critical factor that regulates proliferation of vascular smooth muscle cells in the development of pulmonary vascular remodeling.

Inhibition of the miRNA family miR-130 was achieved by anti-miRs, that were conventionally designed directed against one single member of the miRNA family or by employing LNA seed blockers that have the potential to silence the whole family of miRNAs that share the

same seed region at once. In our experiments, the use of LNA seed blockers showed similar efficacy as anti-miRs. As discussed elsewhere [16], however, LNA seed blockers have the advantage of being active at low (nanomolar) doses and, although they lack tissue specificity and accumulate in many cells, they appear to have no significant side effects. After treatment of hypoxic animals with LNA seed blockers, differences in right heart hypertrophy and in the expression of CDKN1A in lung tissue were no longer significant between these mice and normoxic controls. These data highlight the therapeutic potential of LNA seed blockers to lower the expression of miR-130 *in vivo* and might offer a feasible approach to address proliferation and vascular remodeling in PH.

Taken together, this is the first report emphasizing that the cell-cycle regulator CDKN1A is a direct target of the miRNA family miR-130, which, induced by hypoxia, might be involved in the development of vascular remodeling in PH.

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Conflict of Interest

None declared

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Figure Legends

Figure 1. miR-130 family members are increased in hypoxia-induced Pulmonary Hypertension. (a) In hypoxic animals, the planimetric ratio between the right and left ventricle size including septum indicated right ventricular hypertrophy and was found to be significantly higher than in normoxic controls. The expression levels of miR-130a (b), miR-130b (c), miR-301a (d), and miR-301b (e) were found to be increased in lung tissue of hypoxia-treated mice (10% O_2 for 3 weeks) when compared to normoxic controls. A lower C_t value indicates a higher expression level of the miRNA of interest. Statistical analysis by unpaired Student's t-test (*p<0.05, **p<0.01, ***p<0.001)

Figure 2. The miR-130 family regulates the proliferation of human pulmonary artery smooth muscle cells (HPASMC). (a) HPASMC transfected with precursor molecules for miR-130a (pre-miR-130a), miR-301a (pre-miR-301a) and a cocktail of both showed higher proliferation rates when compared to scrambled transfection (n≥6). (c) In contrast, the inhibition of miRNAs by treatment with individual antisense molecules (anti-miRs) resulted in lower proliferation rates (n≥6). (c) The simultaneous inhibition of all members of the miR-130 family by transfection of LNA seed blockers (LNA anti-miR-130) significantly decreased the proliferation of HPASMC when compared to scrambled negative control (n=9). Statistical analysis by unpaired Student's t-test (*p<0.05, **p<0.01, ***p<0.001).

Figure 3. The tumour suppressor p21 (CDKN1A) is targeted by members of the miR-130 family in human pulmonary artery smooth muscle cells. Enforced expression of miR-130a and miR-301a by transfection with the respective pre-miRs resulted in downregulation of mRNA (a) and protein (b) levels of CDKN1A (n≥4). Conversely, the simultaneous inhibition of all members of the miR-130 family using LNA anti-miR-130 led to upregulation of CDKN1A on the level of mRNA (c) and protein (d) when compared to cells transfected with scrambled negative control (n≥4). Statistical analysis by unpaired (b and d) and paired (a and c) student's t-test (*p<0.05, **p<0.01). The negative control (scrambled) was arbitrarily set as 1 (a and c).

Figure 4. Reporter gene assay confirms a direct and specific regulation of CDKN1A by the miR-130 family. (a) The entire 3'UTR of CDKN1A comprising one miR-130 binding site (seed match) was cloned into a dual luciferase-expressing vector. The pairing of RNA as predicted by TargetScan (www.targetscan.org) between miR-130a and the 3'UTR of CDKN1A (CDKN1A_WT) is shown. In addition, the predicted miR-130 seed match was deleted by introducing three point mutations (construct CDKN1A_ΔmiR130). (b) Reporter gene studies showed that co-transfection of miR-130a and miR-301a significantly lowered the relative luciferase activity of CDKN1A_WT as compared to scrambled transfection (n≥7). The mutated construct was not affected by overexpression of miR-130a and miR-301a (n=4). (c) Conversely, treatment with LNA anti-miR-130 significantly increased the relative luciferase activity of the CDKN1A_WT, whereas no effects on the luciferase activity were observed in the mutated construct (n=5). Statistical analysis by paired Student's t-test (*p<0.05, **p<0.01).

Figure 5. Specific silencing of CDKN1A increases proliferation of human pulmonary artery smooth muscle cells (HPASMC). The expression of CDKN1A was specifically decreased by transfecting HPASMC with gene-specific siRNA (si_CDKN1A). Knockdown of the expression of CDKN1A was confirmed on mRNA (a) and protein level (b) (each n=4). (c) The proliferation of HPASMC was found to be enhanced upon transfection with si_CDKN1A as assessed by BrdU incorporation assay (n≥8). Statistical analysis by paired (a) and unpaired Student's t-test (b and c) (*p<0.05, ***p<0.001). The negative control (scrambled) was arbitrarily set as 1 (a).

Figure 6. Inhibition of the miR-130 family by LNA seed blockers restores the expression of CDKN1A and ameliorates right ventricular hypertrophy in mice. The hypoxia-induced Pulmonary Hypertension mouse model was used to investigate the therapeutical effects of LNA seed blockers. (a) Male mice were randomly separated to receive normoxia saline (n=8), hypoxia saline (n=8), hypoxia LNA scrambled (LNA scr, n=7), and hypoxia LNA anti-miR-130 (LNA 130, n=7). After 21 and 28 days of hypoxia (10% O_2) saline, LNA scr, and LNA 130 (both 2.5mg/kg bodyweight) were intraperitoneally injected. Mice were sacrificed at day 35. (b) The planimetric ratio of the right to left ventricular size was found significantly increased in the hypoxic control group (saline and LNA scr) when compared to normoxic mice. These differences were no longer significant after treatment with LNA 130 indicating an improvement of right ventricular hypertrophy in these mice. Figure bar in tissue slides indicates 1mm. (c) The expression of CDKN1A was measured in lung tissue by western blot. Hypoxic conditions significantly decreased the protein levels of CDKN1A in the saline group when compared to normoxic control mice. Conversely, injections of LNA 130 significantly upregulated the expression of CDKN1A when compared to hypoxic control mice demonstrating the beneficial effect of LNA 130 on the expression of CDKN1A. Statistical analysis by Kruskal-Wallis test with Dunns *post hoc* test (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Figure 1

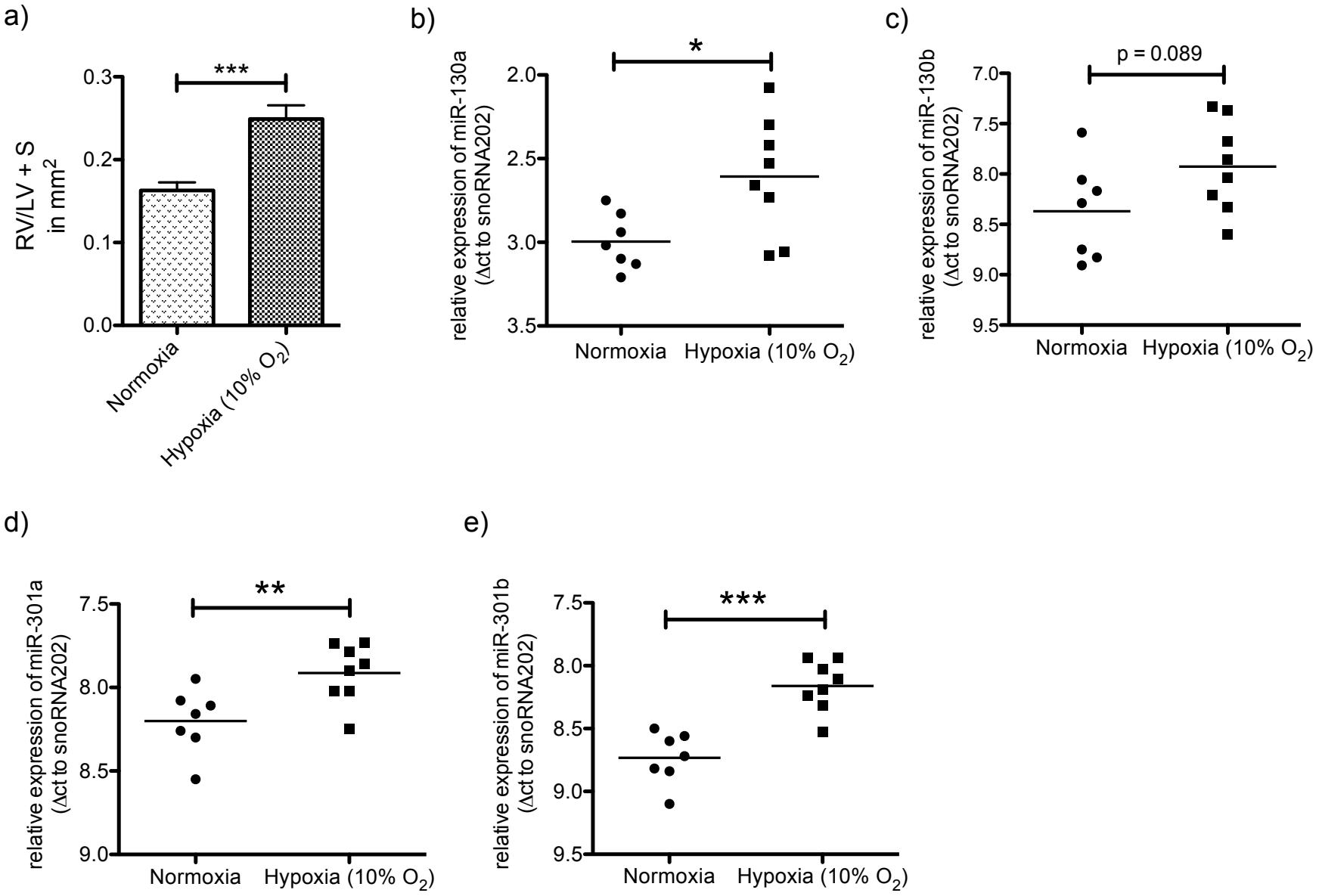

a)
RV/LV + S in mm²
0.3
0.2
0.1
0.0
***
Normoxia
Hypoxia (10% O2)
b)
relative expression of miR-130a (Δct to snoRNA202)
2.0
2.5
3.0
3.5
*
Normoxia
Hypoxia (10% O2)
c)
relative expression of miR-130b (Δct to snoRNA202)
7.0
7.5
8.0
8.5
9.0
9.5
p = 0.089
Normoxia
Hypoxia (10% O2)
d)
relative expression of miR-301a (Δct to snoRNA202)
7.5
8.0
8.5
9.0
**
Normoxia
Hypoxia (10% O2)
e)
relative expression of miR-301b (Δct to snoRNA202)
7.5
8.0
8.5
9.0
9.5
***
Normoxia
Hypoxia (10% O2)

Figure 2

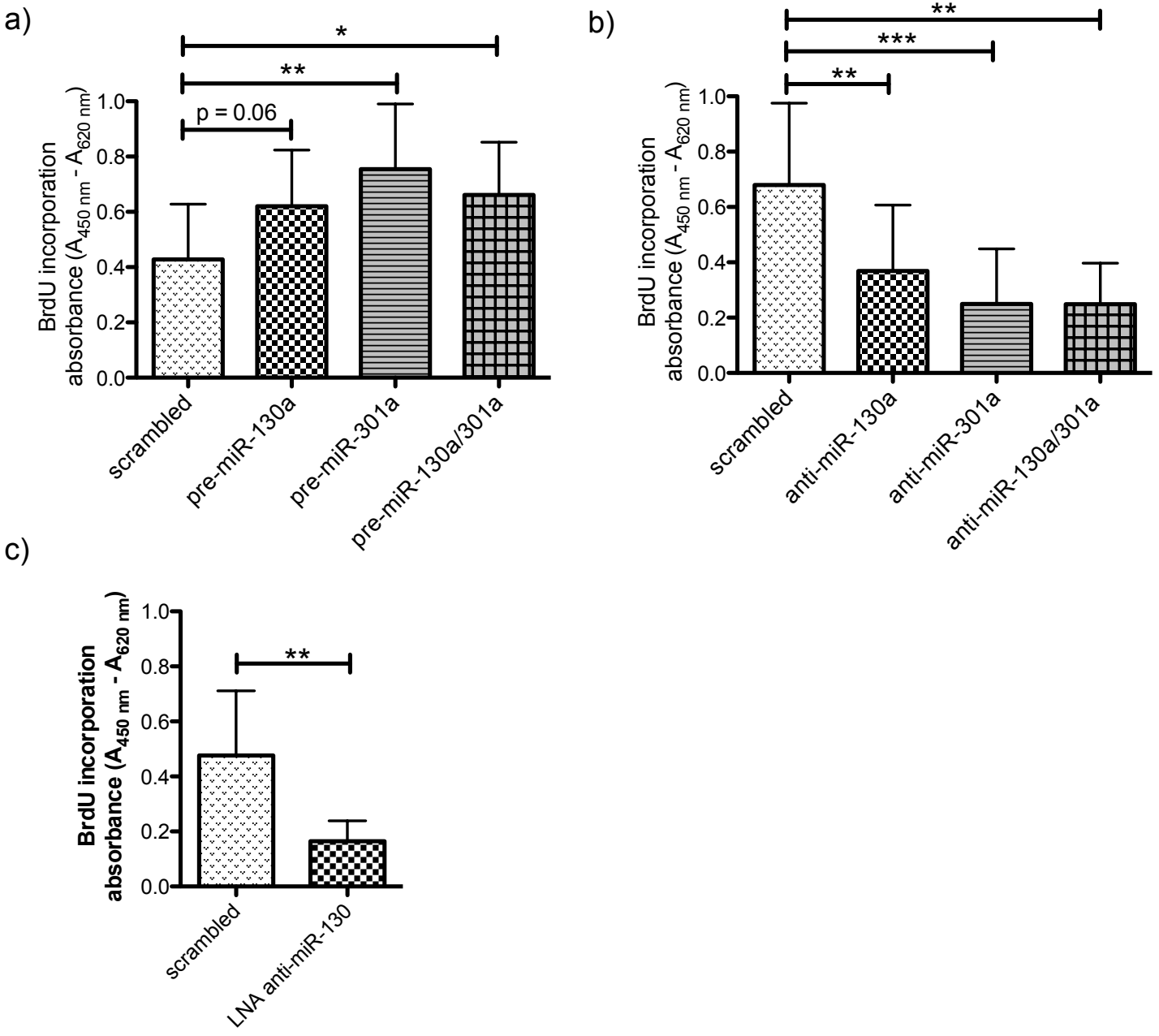

a)
*
**
p = 0.06
BrdU incorporation absorbance ($A_{450\ nm}$ - $A_{620\ nm}$)
1.0
0.8
0.6
0.4
0.2
0.0
scrambled
pre-miR-130a
pre-miR-301a
pre-miR-130a/301a
b)
**
***
**
BrdU incorporation absorbance ($A_{450\ nm}$ - $A_{620\ nm}$)
1.0
0.8
0.6
0.4
0.2
0.0
scrambled
anti-miR-130a
anti-miR-301a
anti-miR-130a/301a
c)
**
BrdU incorporation absorbance ($A_{450\ nm}$ - $A_{620\ nm}$)
1.0
0.8
0.6
0.4
0.2
0.0
scrambled
LNA anti-miR-130

Figure 3

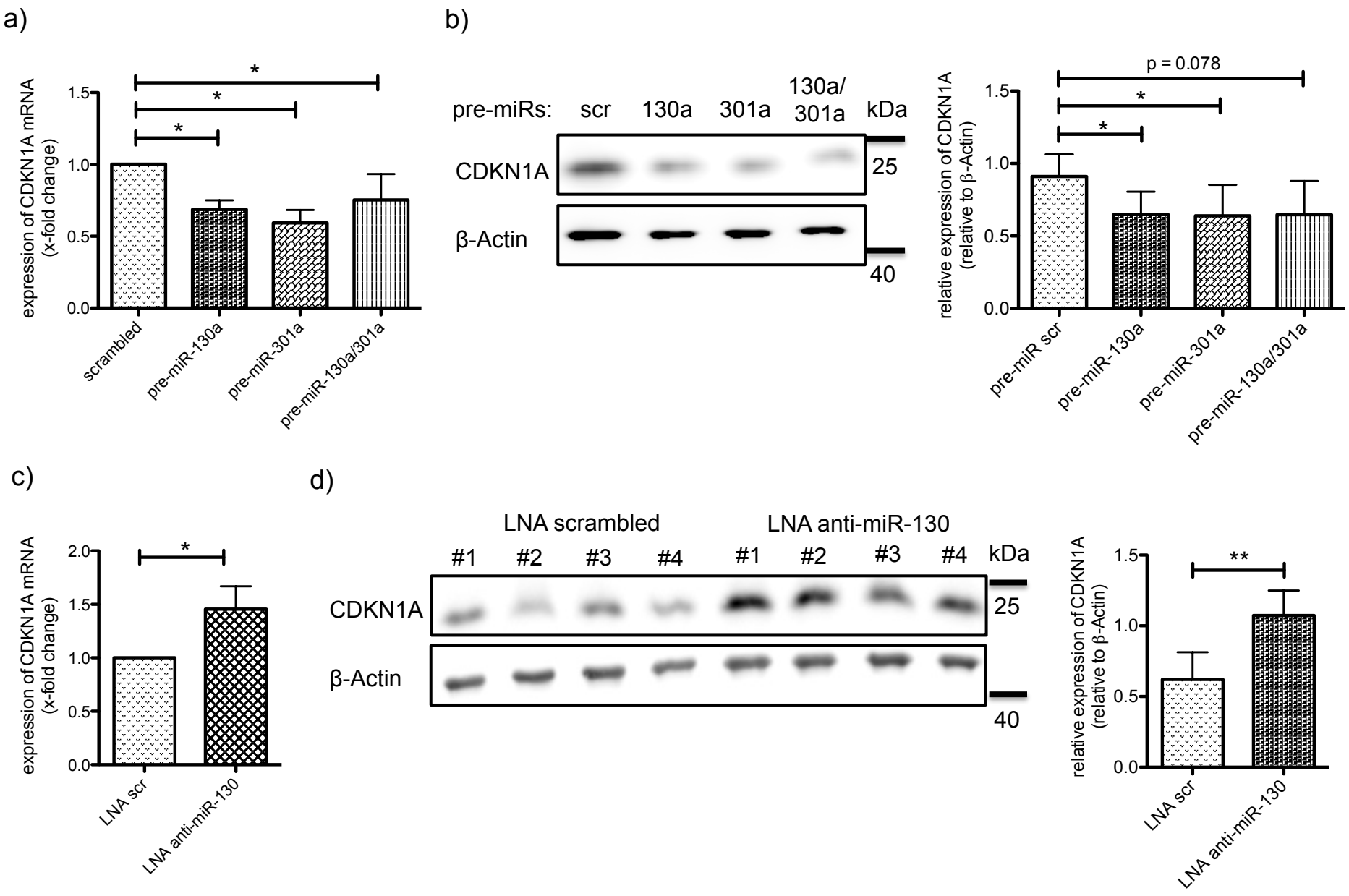

a)
expression of CDKN1A mRNA (x-fold change)
1.5
1.0
0.5
0.0
*
*
*
scrambled
pre-miR-130a
pre-miR-301a
pre-miR-130a/301a
b)
pre-miRs:
scr
130a
301a
130a/ 301a
kDa
CDKN1A
25
β-Actin
40
relative expression of CDKN1A (relative to β-Actin)
p = 0.078
*
*
pre-miR scr
pre-miR-130a
pre-miR-301a
pre-miR-130a/301a
c)
expression of CDKN1A mRNA (x-fold change)
2.0
1.5
1.0
0.5
0.0
*
LNA scr
LNA anti-miR-130
d)
LNA scrambled
LNA anti-miR-130
#1
#2
#3
#4
#1
#2
#3
#4
kDa
CDKN1A
25
β-Actin
40
relative expression of CDKN1A (relative to β-Actin)
**
LNA scr
LNA anti-miR-130

Figure 4

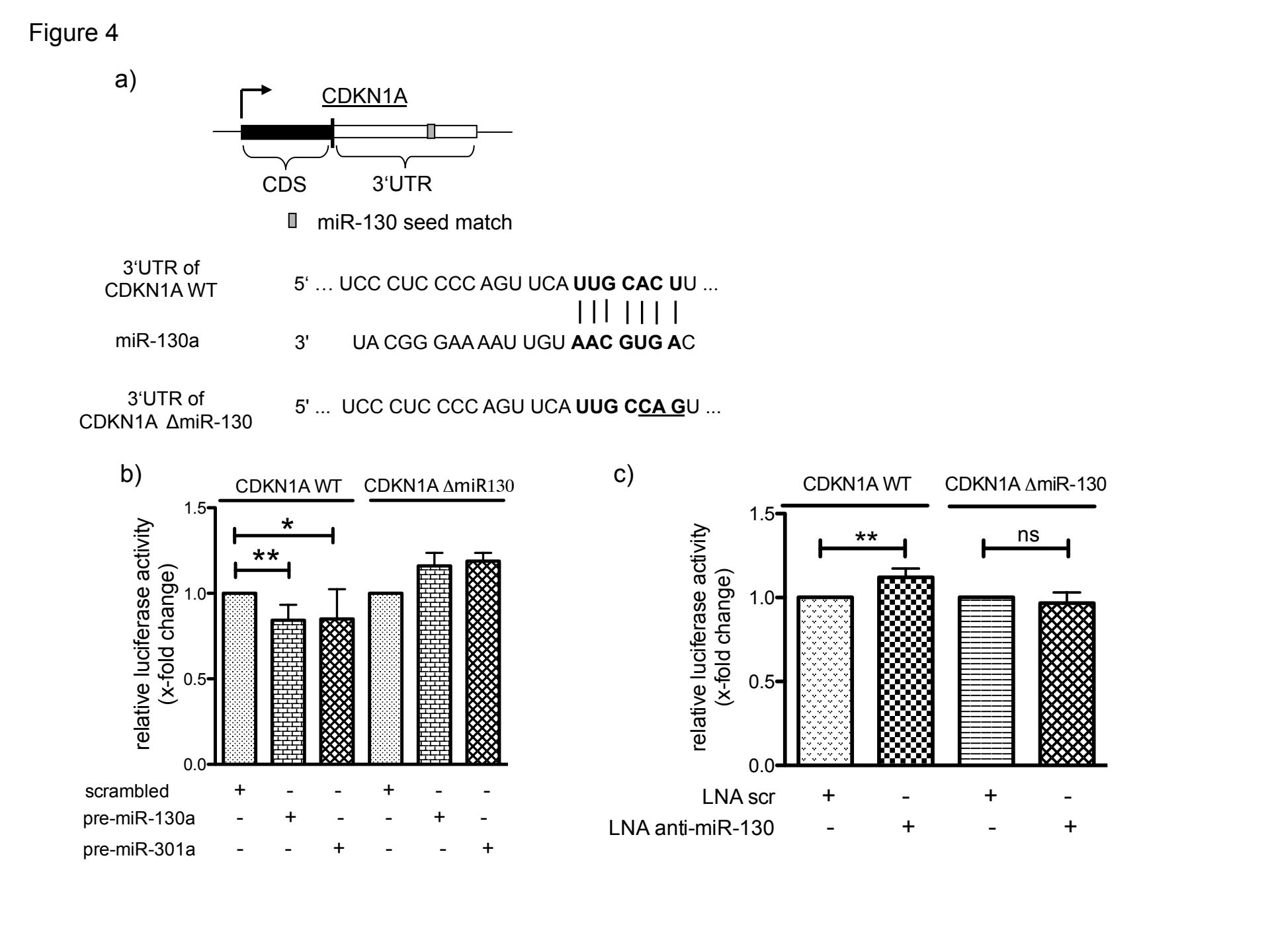

a)
CDKN1A
CDS
3'UTR
miR-130 seed match
3'UTR of CDKN1A WT
5' … UCC CUC CCC AGU UCA UUG CAC UU ...
miR-130a
3' UA CGG GAA AAU UGU AAC GUG AC
3'UTR of CDKN1A ΔmiR-130
5' ... UCC CUC CCC AGU UCA UUG CCA GU ...
b)
CDKN1A WT
CDKN1A ΔmiR130
relative luciferase activity (x-fold change)
1.5
1.0
0.5
0.0
*
**
scrambled + - - + - -
pre-miR-130a - + - - + -
pre-miR-301a - - + - - +
c)
CDKN1A WT
CDKN1A ΔmiR-130
relative luciferase activity (x-fold change)
1.5
1.0
0.5
0.0
**
ns
LNA scr + - + -
LNA anti-miR-130 - + - +

Figure 5

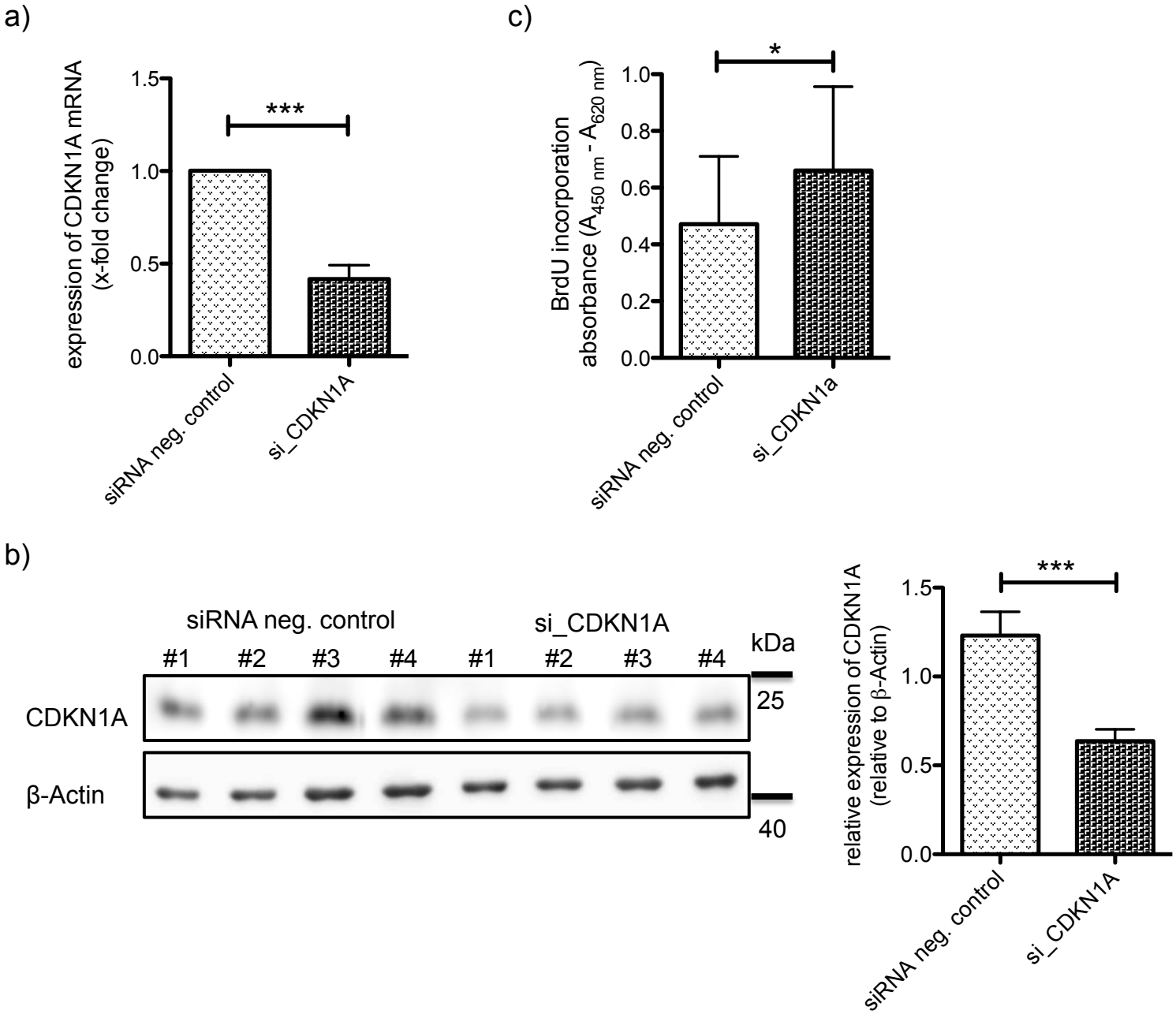
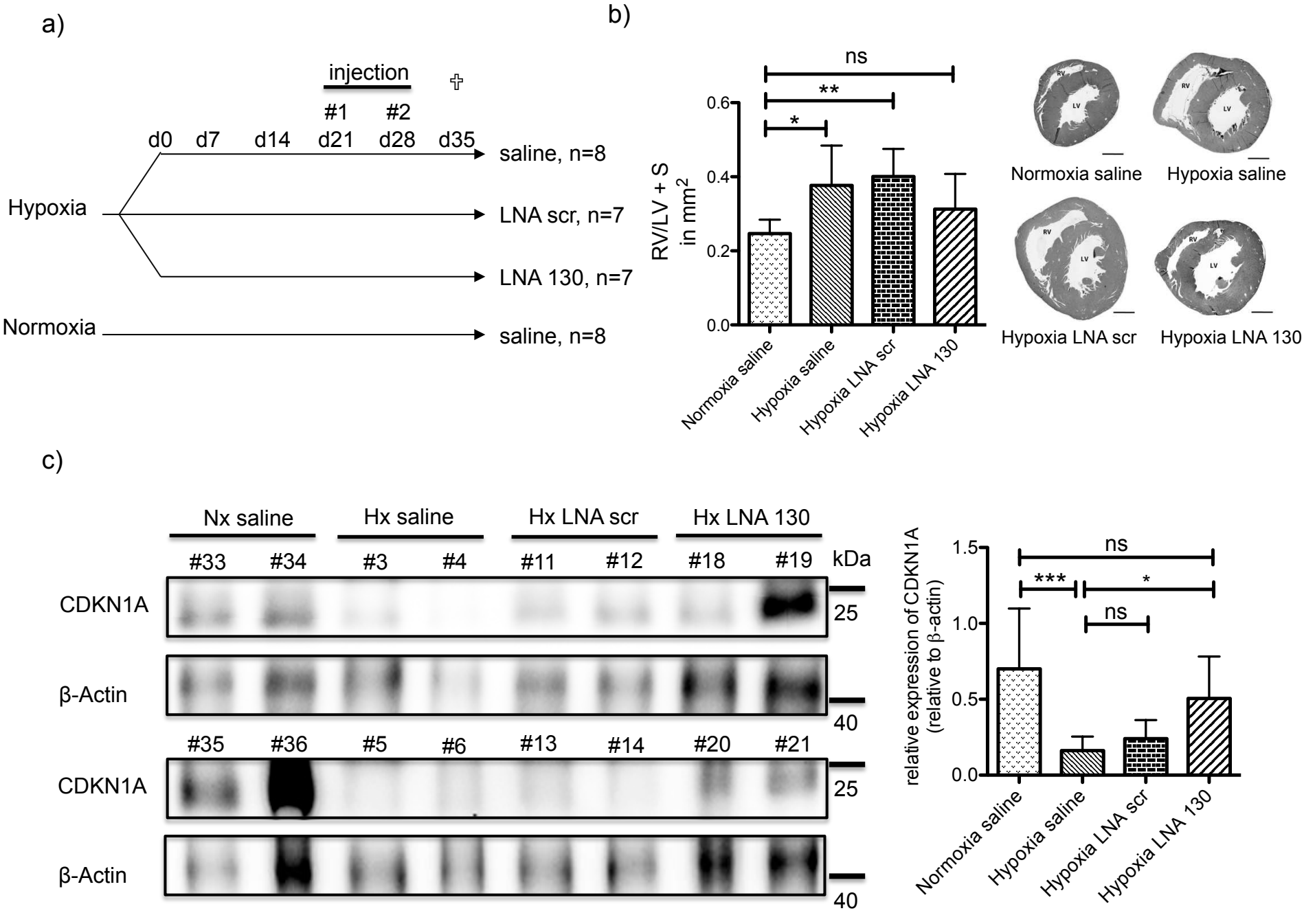

a)
expression of CDKN1A mRNA (x-fold change)
1.5
1.0
0.5
0.0
***
siRNA neg. control
si_CDKN1A
c)
BrdU incorporation absorbance ($A_{450\ nm}$ - $A_{620\ nm}$)
1.0
0.8
0.6
0.4
0.2
0.0
*
siRNA neg. control
si_CDKN1a
b)
siRNA neg. control
si_CDKN1A
#1 #2 #3 #4 #1 #2 #3 #4
kDa
CDKN1A
25
β-Actin
40
relative expression of CDKN1A (relative to β-Actin)
1.5
1.0
0.5
0.0
***
siRNA neg. control
si_CDKN1A

Figure 6


a)
injection
#1
#2
d0
d7
d14
d21
d28
d35
Hypoxia
saline, n=8
LNA scr, n=7
LNA 130, n=7
Normoxia
saline, n=8
b)
ns
**
*
RV/LV + S in mm²
0.6
0.4
0.2
0.0
Normoxia saline
Hypoxia saline
Hypoxia LNA scr
Hypoxia LNA 130
RV
LV
c)
Nx saline
Hx saline
Hx LNA scr
Hx LNA 130
#33
#34
#3
#4
#11
#12
#18
#19
kDa
CDKN1A
25
β-Actin
40
#35
#36
#5
#6
#13
#14
#20
#21
CDKN1A
25
β-Actin
40
relative expression of CDKN1A (relative to β-actin)
1.5
1.0
0.5
0.0
ns
***
*
ns
Normoxia saline
Hypoxia saline
Hypoxia LNA scr
Hypoxia LNA 130

The hypoxia-induced microRNA-130a controls pulmonary smooth muscle cell proliferation by directly targeting CDKN1A.

Appendix

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Table A.1. Primer sequences

miRNA expression primer	
miR-130a/b RT	5’ - GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA GAT CTG ACG ACA TGC CC - 3’
miR-130a fwd (hsa, mmu)	5’ - CTG CAG TGC AAT GTT AAA AGG - 3’
miR-130b fwd (hsa, mmu)	5’ – CAG CAG TGC AAT GAT GAA AGG – 3’
miR-301a/b RT	5’ - GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG CTT TG - 3’
miR-301a fwd (hsa, mmu)	5’ - CCA GTG CAA TAG TAT TGT CAA AG - 3’
miR-301b fwd (hsa)	5’ - GGA GTG CAA TGA TAT TGT CAA AG - 3’
miR-301b fwd (mmu)	5’ - GGA GTG CAA TGG TAT TGT CAA AG - 3’
snoRNA202 RT	5’ – GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC ATC AG – 3’
snoRNA202 (mmu) fwd	5’ – CCG TAC TTT TGA ACC CTT TTC – 3’
RNU48 RT	5’ – GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG GTC AG – 3’
RNU48 (hsa) fwd	5’ – CCA TGA GTG TGT CGC TGA TG – 3’
RNU49 RT	5’ – GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA ATC AG – 3’
RNU49 (hsa) fwd	5’ – AAG CGA TAA CTG ACG AAG ACT AC – 3’
universal reverse primer	5’ - GAG GTA TTC GCA CTG GAT AC - 3’
gene expression primer	
β-actin (hsa, mmu) fwd	5’ – TCA AGA TCA TTG CTC CTC CTG AG – 3’
β-actin (hsa, mmu) rev	5’ – TCC TGC TTG CTG ATC CAC ATC – 3’
CDKN1A (mmu) fwd	5’ - AGC CTG ACA GAT TTC TAT CAC TC – 3’
CDKN1A (mmu) rev	5’ - AGG GTT TTC TCT TGC AGA AGA C – 3’
CDKN1A (hsa) fwd	5’ - AGC ATG ACA GAT TTC TAC CAC TC – 3’
CDKN1A (hsa) rev	5’ - GGC TTC CTC TTG GAG AAG ATC – 3’
cloning primer	
3’UTR of CDKN1A (XbaI) fwd	5’ - CGA GTC TAG ATC CGC CCA CAG GAA GC – 3’
3’UTR of CDKN1A (SalI) rev	5’ - CCT GCA GGT CGA CAC AAG TAA AGT CAC TAA GAA TC – 3’
3’UTR of CDKN1A (ΔmiR-130) fwd	5’ – ATT GCC AGT TGA TTA GCA GCG – 3’
3’UTR of CDKN1A (ΔmiR-130) rev	5’ – ATC AAC TGG CAA TGA ACT GGG – 3’

Appendix Figure Legends

Figure A. 1. Kinetics of miR-130a expression *in vivo*. Expression levels of miR-130a in lung tissue from mice exposed to 10% oxygen for 2 (n=3), 3 (n=8) and 5 (n=8) weeks are shown. Concomitant invasive hemodynamic measurement (right ventricular systolic pressure, RSVP) and planimetric assessment of volume changes of the right ventricle are provided below. Statistical analysis by unpaired Student's t-test (*p<0.05).

Figure A. 2. Hypoxia induces the expression of miR-130a and miR-301a in human pulmonary artery smooth muscle cells (HPASMC). (a) HPASMC cultured in hypoxic conditions showed higher expression of miR-130a and miR-301a in a time-dependent manner when compared to normoxic cells (n=4). (b) Hypoxia had no effect on the expression of miR-130 family members in human pulmonary artery endothelial cells (n=5). Statistical analysis by paired Student's t-test (**p<0.01). The dashed line indicates the expression levels of normoxic control cells, which was arbitrarily set as 1.

Figure A. 3. Viability and apoptosis assay. (a) Viability of HPASMC following overexpression of miR-130, miR-301a and a cocktail of both was tested by using the MTT-based CellTiter assay and showed a significant increase of cell viability in miR-301a and cocktail transfected cells (n=4). (b) Conversely, no effects were observed on apoptosis of HPASMC after overexpression of miR-130a, miR-301a and a cocktail of both as analysed by the activity of caspase 3/ 7 (n=4). Statistical analysis by unpaired Student's t-test (*p<0.05).

Figure A. 4. Confirmation of successful transfection of the miR-130 family in human pulmonary artery smooth muscle cells (HPASMC). (a) The expression of miR-130a was significantly increased upon transfection of HPASMC with pre-miR as measured by qPCR (n=4). (b) In contrast, the levels of miR-130a did not change when LNA seed blockers (LNA anti-miR-130) were transfected (n=4). Seed blockers sequestrate their target miRNA by forming a heteroduplex structure whereas the stability of the miRNA is not affected [1]. Similar results were obtained when the expression of miR-301a was measured in pre-miR (c) or in LNA anti-miR-130 (d) transfected HPASMC (n=4). **p<0.01, ***p<0.001 by paired Student's t-test. The negative control (scrambled) was arbitrarily set as 1.

Figure A. 5. The expression of CDKN1A is repressed by hypoxia *in vitro* and *in vivo*. (a) Levels of CDKN1A mRNA in human pulmonary artery smooth muscle cells kept under hypoxic conditions (1% O_2) were found to be significantly decreased at different time points (n=4). (b) Similarly, mRNA levels of CDKN1A in hypoxic mice (3 weeks at 10% O_2, n=8) were significantly lower when compared to normoxic control animals (n=7). *p<0.05, **p<0.01 by paired Student's t-test. The dashed line indicates the expression levels of normoxic control cells, which was arbitrarily set as 1 (a).

Figure A. 6. Design of seed blockers directed against the seed sequence of the miR-130 family. The sequence of four members of the miR-130 family (miR-130a, miR-130b, miR-301a, miR-301b) is shown. The LNA seed blockers were specifically designed to bind to the seed region of miR-130, a sequence motif shared by all members of the miRNA family.

References

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Figure A.1

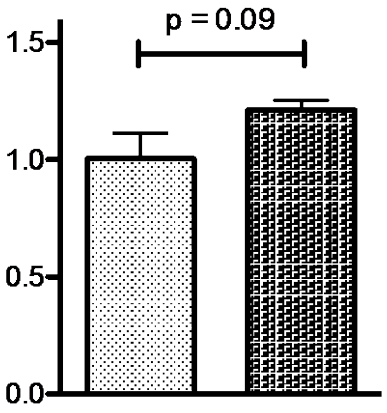
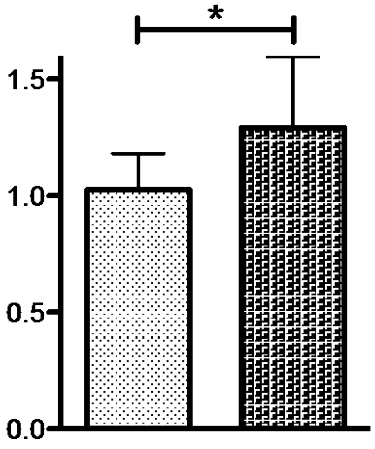
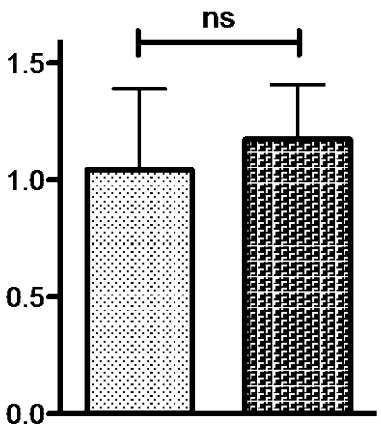
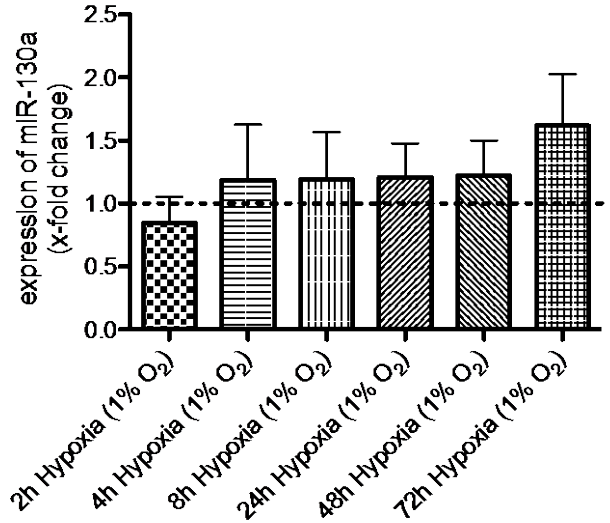
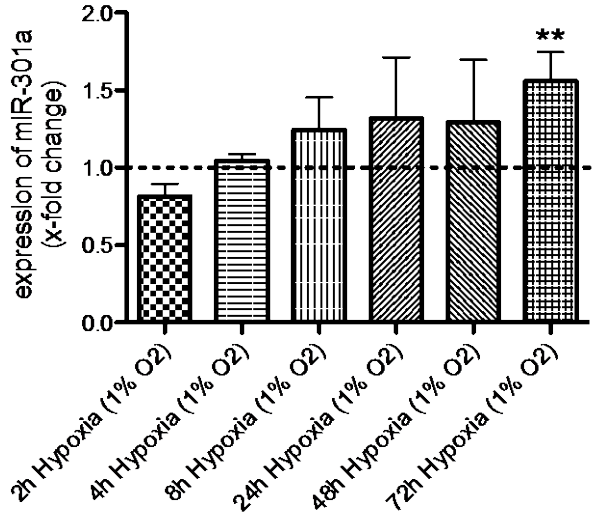
	2 weeks		3 weeks		5 weeks	
	Nx	Hx	Nx	Hx	Nx	Hx
Expression of miR-130a (x-fold change)	 1 ± 0.11	1.21 ± 0.04	 1.03 ± 0.15	1.29 ± 0.31	 1.04 ± 0.35	1.18 ± 0.23
RVSP (mmHg)	27	40.5	-	-	30.83 ± 2.6	45.5 ± 3.63
RV/ (LV+S) (mm^2)	-	-	0.16 ± 0.01	0.25 ± 0.02	0.25 ± 0.04	0.38 ± 0.11

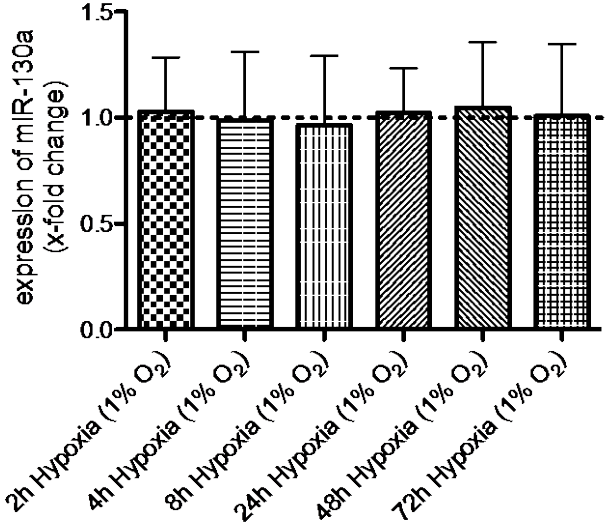
Figure A.2

a)


expression of miR-130a
(x-fold change)
2.5
2.0
1.5
1.0
0.5
0.0
2h Hypoxia (1% O2)
4h Hypoxia (1% O2)
8h Hypoxia (1% O2)
24h Hypoxia (1% O2)
48h Hypoxia (1% O2)
72h Hypoxia (1% O2)


expression of miR-301a
(x-fold change)
2.0
1.5
1.0
0.5
0.0
**
2h Hypoxia (1% O2)
4h Hypoxia (1% O2)
8h Hypoxia (1% O2)
24h Hypoxia (1% O2)
48h Hypoxia (1% O2)
72h Hypoxia (1% O2)

b)


expression of miR-130a
(x-fold change)
1.5
1.0
0.5
0.0
2h Hypoxia (1% O2)
4h Hypoxia (1% O2)
8h Hypoxia (1% O2)
24h Hypoxia (1% O2)
48h Hypoxia (1% O2)
72h Hypoxia (1% O2)

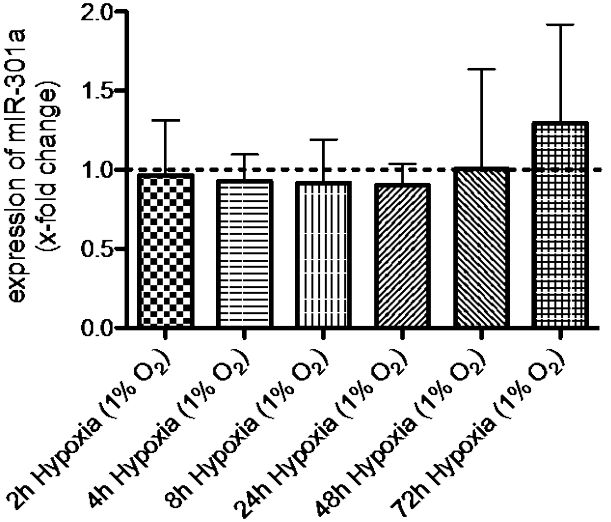
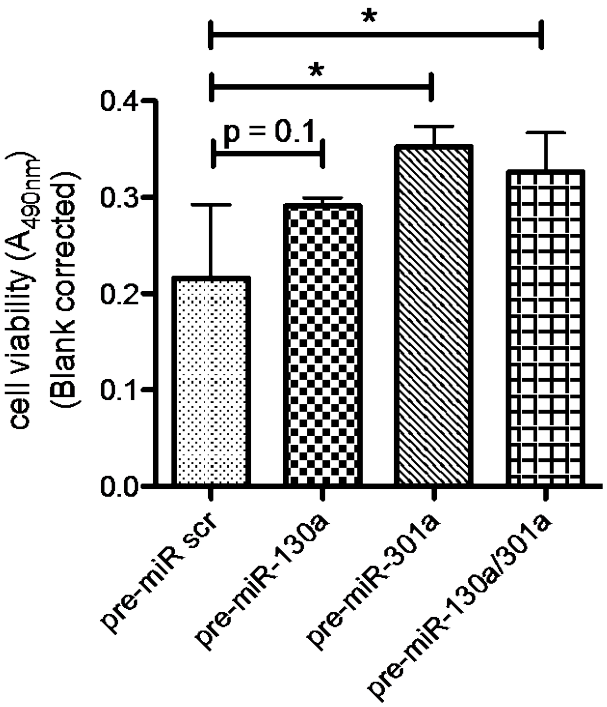

expression of miR-301a
(x-fold change)
2.0
1.5
1.0
0.5
0.0
2h Hypoxia (1% O2)
4h Hypoxia (1% O2)
8h Hypoxia (1% O2)
24h Hypoxia (1% O2)
48h Hypoxia (1% O2)
72h Hypoxia (1% O2)

Figure A.3

a)


*
*
p = 0.1
0.4
0.3
0.2
0.1
0.0
cell viability (A_{490nm})
(Blank corrected)
pre-miR scr
pre-miR-130a
pre-miR-301a
pre-miR-130a/301a

b)

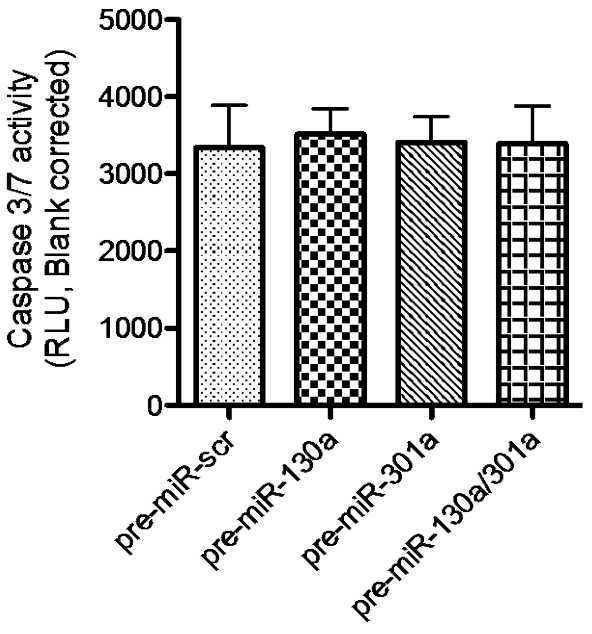

5000
4000
3000
2000
1000
0
Caspase 3/7 activity
(RLU, Blank corrected)
pre-miR-scr
pre-miR-130a
pre-miR-301a
pre-miR-130a/301a

Figure A.4

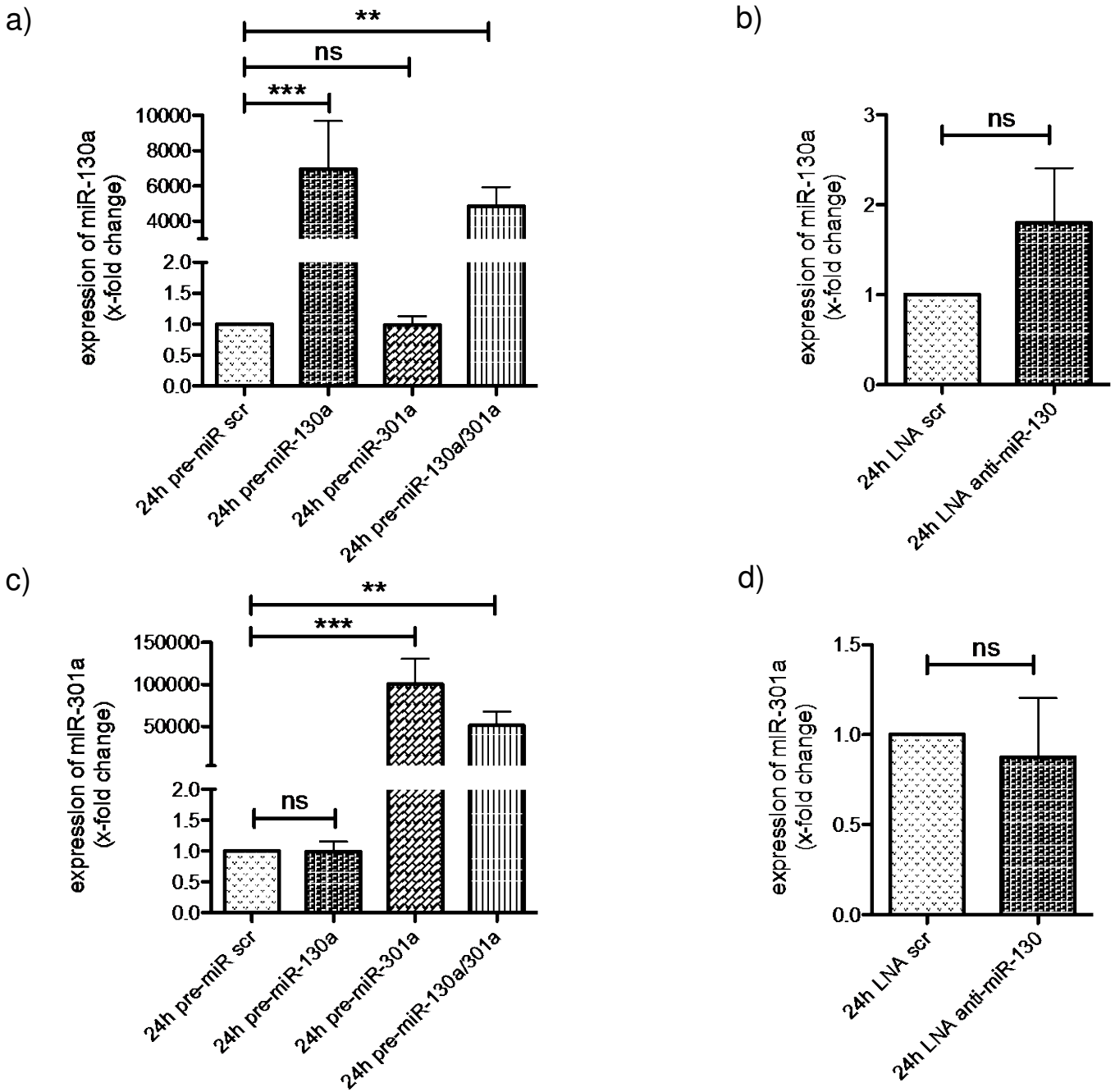
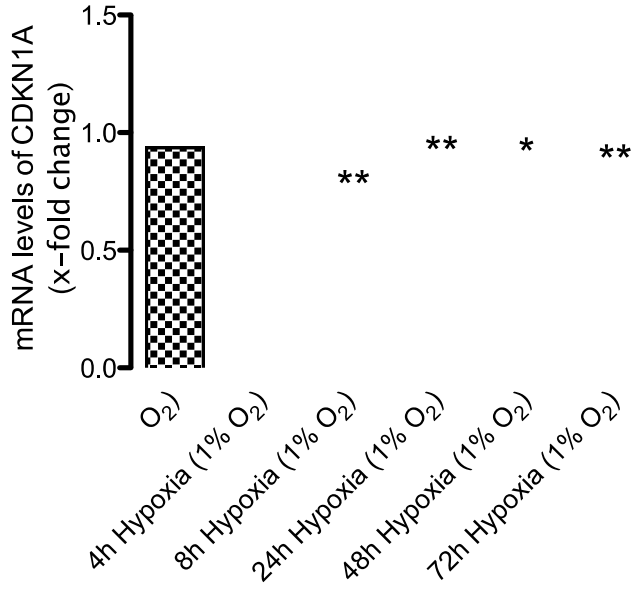

a)
**
ns
***
expression of miR-130a (x-fold change)
10000
8000
6000
4000
2.0
1.5
1.0
0.5
0.0
24h pre-miR scr
24h pre-miR-130a
24h pre-miR-301a
24h pre-miR-130a/301a
b)
ns
expression of miR-130a (x-fold change)
3
2
1
0
24h LNA scr
24h LNA anti-miR-130
c)
**
***
expression of miR-301a (x-fold change)
150000
100000
50000
ns
2.0
1.5
1.0
0.5
0.0
24h pre-miR scr
24h pre-miR-130a
24h pre-miR-301a
24h pre-miR-130a/301a
d)
ns
expression of miR-301a (x-fold change)
1.5
1.0
0.5
0.0
24h LNA scr
24h LNA anti-miR-130

Figure A.5

a)


mRNA levels of CDKN1A
(x-fold change)
1.5
1.0
0.5
0.0
**
**
*
**
O2)
4h Hypoxia (1% O2)
8h Hypoxia (1% O2)
24h Hypoxia (1% O2)
48h Hypoxia (1% O2)
72h Hypoxia (1% O2)

b)

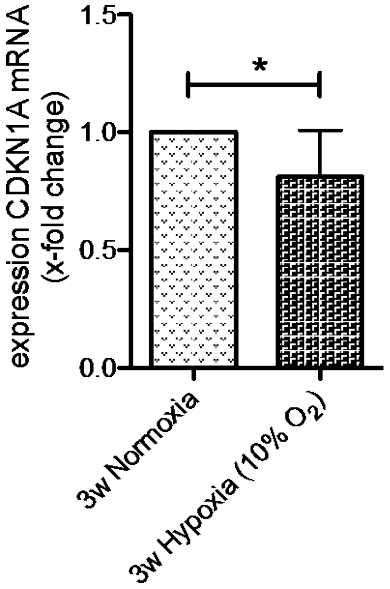

expression CDKN1A mRNA
(x-fold change)
1.5
1.0
0.5
0.0
*
3w Normoxia
3w Hypoxia (10% O2)

Figure A.6

miRNA family miR-130

miR-130a	5’ - C**AG UGC AAU** GUU AAA AGG GCA U - 3’
miR-130b	5’ - C**AG UGC AAU** GAU GAA AGG GCA U - 3’
miR-301a	5’ - C**AG UGC AAU** AGU AUU GUC AAA GC - 3’
miR-301b	5’ - C**AG UGC AAU** GAU AUU GUC AAA GC - 3’
Seed region:	**AG UGC AAU**
Seed blocker:	3’ - **UC ACG UUA - 5’**